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Real-Time Polymerase Chain Reaction Assay for Cell-Associated HTLV Type I DNA Viral Load

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ABSTRACT

We have developed a quantitative real-time PCR assay for HTLV-I DNA. This assay approach uses real-time monitoring of fluorescent signal generation as a consequence of *Taq*-mediated amplification of specific target sequences to allow real-time kinetic analysis of amplicon production. This kinetic approach yields excellent sensitivity and an extremely broad linear dynamic range, and ensures that quantitation is based on analysis during the exponential phase of amplification, regardless of the input template copy number. The HTLV-I DNA assay has a nominal threshold sensitivity of 10 copy Eq/reaction, although single-copy plasmid template can be detected at frequencies consistent with statistical prediction. The linear dynamic range is in excess of 5 logs. Interassay reproducibility averages 14% (coefficient of variation) for control templates over a range of 10^1 to 10^6 copy Eq/reaction and 25%, based on studies of extraction and analysis of replicate aliquots of PBMC specimens from HTLV-I-infected subjects. The primer/probe combination targets *tax* sequences conserved across described HTLV-I and HTLV-II isolates. Parallel quantitation in the same samples of an endogenous sequence present at a known copy number per cell allows normalization of results for potential variation in DNA recovery. Availability of this assay should facilitate studies of basic pathogenesis and clinical evaluation of HTLV-I and HTLV-II infection, as well as assessment of therapeutic approaches.

INTRODUCTION

HTLV-I WAS THE FIRST human retrovirus to be identified[1] and has been etiologically linked to adult T cell leukemia/lymphoma (ATLL[2,3]) and to nonneoplastic inflammatory diseases, including HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis, and infective dermatitis of children (reviewed in Refs. 4–7). HTLV-I infection has also been implicated in an increasing spectrum of disease manifestations and may have a potential contributory role in the pathogenesis of a number of other clinical syndromes (reviewed in Refs. 4–7). The worldwide distribution and seroepidemiology of HTLV-I infection have been well established, with identification of endemic populations in Asia, Africa, certain Pacific islands, South America, and the Mediterranean region, along with immigrant populations with increased seroprevalence in the United States and Europe, originating from endemic populations (reviewed in Refs. 4–7). However, beyond the demonstrated capability of HTLV-I to transform human lymphocytes *in vitro*,[8,9] and to induce expansion of oligoclonal populations of T lymphocytes *in vivo*,[10–13] the mechanisms of pathogenesis for HTLV-I-associated diseases remain somewhat obscure, particularly in view of the relatively low frequency of overt disease manifestations among seropositive individuals.

The development of quantitative assays for retroviral nucleic acids has revolutionized the study of human immunodeficiency virus (HIV) infection,[14–18] and related animal model systems.[19–23] Quantitative analysis of viral RNA levels, in partic-

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ular plasma RNA levels, has led to revision of basic paradigms of pathogenesis,[17,24,25] improved understanding of prognosis,[26,27] better and more rapid evaluation of experimental therapies,[28] and enhanced monitoring of clinical status.[29] However, in contrast to HIV-1, cell-free viremia is not a prominent aspect of HTLV-associated diseases. Indeed, infection of susceptible target cells *in vitro* with cell-free virus has been achieved only with great difficulty, with cocultivation approaches used more commonly[8,9] prior to the availability of infectious molecular clones for transfection.[30] Accordingly, some investigators have sought to quantify cell-associated HTLV-I DNA viral load, but until recently the methods used have been comparatively cumbersome, or have suffered from other limitations.[31–37]

Newly developed technology allows real-time quantitative analysis of product accumulation during ongoing polymerase chain reaction (PCR) amplifications. This approach obviates many of the difficulties inherent in attempting to use PCR for quantitative applications.[22,38,39] Using this approach, we and others have developed quantitative real-time PCR and reverse transcriptase (RT)-PCR assays for a variety of target templates, including specific retroviral nucleic acid species.[22,40] Here we describe a quantitative real-time PCR assay for HTLV-I DNA. The assay has excellent sensitivity, linear dynamic range, precision, and accuracy, and encompasses numerous practical advantages over other approaches. On the basis of targeting sequences highly conserved between HTLV-I and HTLV-II, the assay should provide for rapid, accurate, and convenient quantitation of HTLV-I and HTLV-II viral load in clinical specimens, greatly facilitating studies of the role of viral replication in the pathogenesis of HTLV-I- and HTLV-II-associated diseases.

MATERIALS AND METHODS

Prism 7700 sequence detector system: General principles

The instrumentation and underlying chemistry of the Prism 7700 sequence detector system (Perkin-Elmer/Applied Biosystems, Foster City, CA), and its operating principles have been described in detail elsewhere, along with an approach for application of this system to the quantitation of viral nucleic acids.[22,38–40]

Purification and characterization of control templates

As a control template, we used the plasmid pMT2 containing the full-length HTLV-I Tax-coding region in pUC13.[41] The plasmid was propagated in *Escherichia coli* DH5α cells and the DNA was purified chromatographically (Qiagen, Sanata Clara, CA). Quantification of the linearized purified template was by A_{260} measurements, on the basis of the calculated extinction coefficient for the insert-containing plasmid sequence. Limiting dilution PCR titered to end point in the appropriate range for the calculated copy number (data not shown).

PCR primers and labeled hybridization probe

The primers used were HTV-F5 (5′-CGG ATA CCC IGT CTA CGT GTT T-3′; HTLV-I nucleotides 7359 to 7380 [GenBank accession number J02029[42]], HTLV-II nucleotides 7248 to 7269 [GenBank accession number M10060[43,44]]) and HTV-R4 (5′-CTG AGC IGA IAA CGC GTC CA-3′; HTLV-I nucleotides 7519 to 7500, HTLV-II nucleotides 7408 to 7389. Inosine residues (I) were incorporated into the primers to avoid misquantitation due to sequence mismatches at positions of known sequence divergence among sequenced isolates.[16] The probe used was P-HTV-02 (5′-**R**-ATC ACC TGG GAC CCC ATC GAT GGA-**Q**-3′; HTLV-I nucleotides 7480 to 7503, HTLV-II nucleotides 7369 to 7392), where **R** indicates a FAM group and **Q** indicates a TAMRA group conjugated through a linker arm nucleotide linkage (LAN), as described.[45] FAM serves as the reporter fluorochrome and TAMRA serves as the quencher; the probe is 3′-blocked so that it does not itself prime PCR amplification. Double high-performance liquid chromatography (HPLC)-purified probe was obtained from DNA Sciences (San Diego, CA), or from Operon Technologies (Alameda, CA). Figure 1 shows alignments of the primer and probe sequences across a representative range of determined HTLV sequences. Gel analysis confirmed amplification of the correctly sized expected 161-bp amplicon (data not shown).

As a normalization control for DNA recovery, and variations in amplification efficiency, including variations attributable to the presence of substances inhibitory for PCR reactions in isolated DNA samples, some cell-derived DNA specimens were also tested for an endogenous sequence derived from the endogenous retrovirus ERV-3, present at known copy number per diploid cell,[46,47] using primers PHP-10F (5′-CAT GGG AAG CAA GGG AAC TAA TG-3′; nucleotides 1051 to 1073) and PHP-10R (5′-CCC AGC GAG CAA TAC AGA ATT T-3′; nucleotides 1185 to 1164), and probe PHP-P05 (5′-R-TCT TCC CTC GAA CCT GCA CCA TCA AAG TCA-Q-3′; nucleotides 1101 to 1129) (GenBank accession number M12140, all nucleotide position numbers according to Ref. 48) as described by Yuan and Waters.[47]

PCR amplification

For each sample, 10 μl of DNA (control template or test sample, typically corresponding to approximately 1×10^5 cell equivalents of DNA for test samples) was amplified in 96-well arrays of optical-grade thin-wall PCR tubes or 96-well optical plates (Perkin-Elmer), with each 50-μl (final volume) PCR containing the indicated final amounts or concentrations of the following components in RNase/DNase-free water, based on empirical optimization: TaqMan buffer A (1×), dATP, dCTP, dGTP (300 μM each), and dUTP (600 μM), $MgCl_2$ (4 mM) (5 mM for ERV-3 amplifications), primers HTV-F5 and HTV-R4 (or PHP-10F and PHP-10R for ERV-3) (1 μM each), probe pHTV-02 (300 nM) (PHP-P05, 250 nM for ERV-3), AmpliTaq Gold (1.25 U), and uracil *N*-glycosylase (1.0 U) (all reaction components except primers and probe from Perkin-Elmer). After 2 min at 50°C (uracil *N*-glycosylase digestion for contamination control) and 10 min at 95°C (thermal activation of the AmpliTaq Gold for hot-start PCR), 45 cycles of PCR amplification were performed (95°C, 15 sec; 58°C, 60 sec) for both HTLV-I and ERV-3 amplifications.

Prism 7700 assay format

For each run, a standard curve was generated from duplicate samples of $\log_{10}$ dilutions in nuclease-free Tris–EDTA buffer

Genbank #	Designation		Origin
Forward primer F5			
		5′- Cgg ATA CCC igT CTA CgT gTT T-3′	
[D13784]	HTLV-Ia	... ... a..	(ATL isolate-Caribbean origin)
[U19949]	HTLV-Ia	... ... a..	(ATL-YS genome)
[L36905]	HTLV-Ia	... ... a..	(Proviral sequence-uncultured blood)
[L03561]	HTLV-Ia	... ... a..	(Isolate from a rabbit cell line)
[J02029]	HTLV-Ia	... ... a..	(Proviral genome-ATL)
[M69044]	HTLV-Ia	... ... a..	(Env gene-African origin)
[M86840]	HTLV-Ia	... ... a..	(HTLV-I from TSP patient)
[Z46900]	HTLV-Ia	... ... a..	(Isolate from macaque)
[L02534]	HTLV-Ic	... ... t.. a..	(Melanesian isolate-MEL5)
[M10060]	HTLV-IIa	t.. ... ... c..	(Proviral DNA-Infectious Mol. clone)
[U32875]	HTLV-IIa	t.. ... ... c..	(Kayapo Indian from Brazil)
[U32898]	HTLV-IIa	t.. ... ... c..	(IVDA from Sao Paulo, Brazil)
[Y13051]	HTLV-IIa	t.. ... ... c..	(African isolate HTLV-CGE)
[X89270]	HTLV-IIa	t.. ... ... c..	(Italian isolate)
[L11456]	HTLV-IIb	t.. ... ... c..	(Guayami Indian isolate G-12)
[L20734]	HTLV-IIb	t.. ... ... c..	(NRA from Italian blood donor)
[U32882]	HTLV-IIb	t.. ... ... c..	(FUC; IVDA from New York)
Reverse primer R4			
		5′-CTg AgC igA iAA CgC gTC CA-3′	
[D13784]	HTLV-Ia	... ... c.. t..	(ATL isolate-Caribbean origin)
[U19949]	HTLV-Ia	... ... c.. t..	(ATL-YS genome)
[L36905]	HTLV-Ia	... ... c.. t..	(Proviral sequence-uncultured blood)
[L03561]	HTLV-Ia	... ... c.. t..	(Isolate from rabbit cell line)
[J02029]	HTLV-Ia	... ... c.. t..	(Proviral genome-ATL)
[M69044]	HTLV-Ia	... ... c.. t..	(Env gene-African origin)
[M86840]	HTLV-Ia	... ... c.. t..	(HTLV-I from a TSP patient)
[Z46900]	HTLV-Ia	... ... c.. t..	(Isolate from macaque)
[L02534]	HTLV-Ic	... ... c.. t..	(Melanesian isolate-MEL5)
[M10060]	HTLV-IIa	ga. ... t.. c..	(Proviral DNA-Infectious Mol. clone)
[U32875]	HTLV-IIa	ga. ... t.. c..	(Kayapo Indian from Brazil)
[U32898]	HTLV-IIa	ga. ... t.. c..	(IVDA from Sao Paulo, Brazil)
[Y13051]	HTLV-IIa	ga. ... t.. c..	(African isolate HTLV-CGE)
[X89270]	HTLV-IIa	ga. ... t.. c..	(Italian isolate)
[L20734]	HTLV-IIb	ga. ... t.. c..	(Guaymi Indian isolate G-12)
[L20734]	HTLV-IIb	ga. ... t.. c..	(NRA from an Italian blood donor)
[U32882]	HTLV-IIb	ga. ... t.. c..	(FUC; IVDA from New York)
Probe P-HTV-02:			
		5′-ATC ACC Tgg gAC CCC ATC gAT ggA-3′	
[D13784]	HTLV-Ia	... ...	(ATL isolate-Caribbean origin)
[U19949]	HTLV-Ia	... ...	(ATL-YS genome)
[L36905]	HTLV-Ia	... ...	(Proviral sequence-uncultured blood)
[L03561]	HTLV-Ia	... ...	(Isolate from a rabbit cell line)
[J02029]	HTLV-Ia	... ...	(Proviral genome-ATL)
[M69044]	HTLV-Ia	... ...	(African origin-env gene)
[M86840]	HTLV-Ia	... ...	(HTLV-I from a TSP patient)
[Z46900]	HTLV-Ia	... ...	(Isolate from macaque)
[L02534]	HTLV-Ic	... .a	(Melanesian isolate-MEL5)
[M10060]	HTLV-IIa	c..	(Proviral DNA-Infectious Mol. clone)
[U32875]	HTLV-IIa	c..	(Kayapo Indian from Brazil)
[U32898]	HTLV-IIa	c..	(IVDA from Sao Paulo, Brazil)
[Y13051]	HTLV-IIa	c..	(African isolate HTLV-CGE)
[X89270]	HTLV-IIa	c..	(Italian isolate)
[L20734]	HTLV-IIb	c..	(Guaymi Indian isolate G-12)
[L20734]	HTLV-IIb	c..	(NRA from an Italian blood donor)
[U32882]	HTLV-IIb	c..	(FUC; IVDA from New York)

FIG. 1. Alignment of HTLV primers and probe with representative HTLV sequences.

(TE; 10 mM Tris, 0.1 mM EDTA, pH 8.0) of purified pMT2 control template, ranging from 1×10^6 to 1×10^0 nominal copy equivalents/reaction. Fresh dilutions of the control standard were prepared for each experiment from freshly thawed single-use aliquots of a stock preparation stored at −20°C. Threshold cycle (C_t) values were plotted as a function of nominal input template copy number and a least-squares regression performed with the Prism 7700 software.

For each test specimen, triplicate reactions were performed, using 10% of the total extracted DNA per reaction (this typically corresponded to approximately 1×10^5 cell equivalents, or approximately 1 μg of DNA by A_{260} measurement). Nominal copy numbers for test samples were then automatically calculated by interpolation of the experimentally determined C_t value for the test sample onto the control standard regression curve. Assay acceptability was contingent on the regression R^2 value for the standard curve being >0.97.

Specimen preparation/DNA extraction

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque density centrifugation from freshly drawn whole blood and viably cryopreserved until testing. For analysis, cells were thawed, washed, and total cells counted by he-

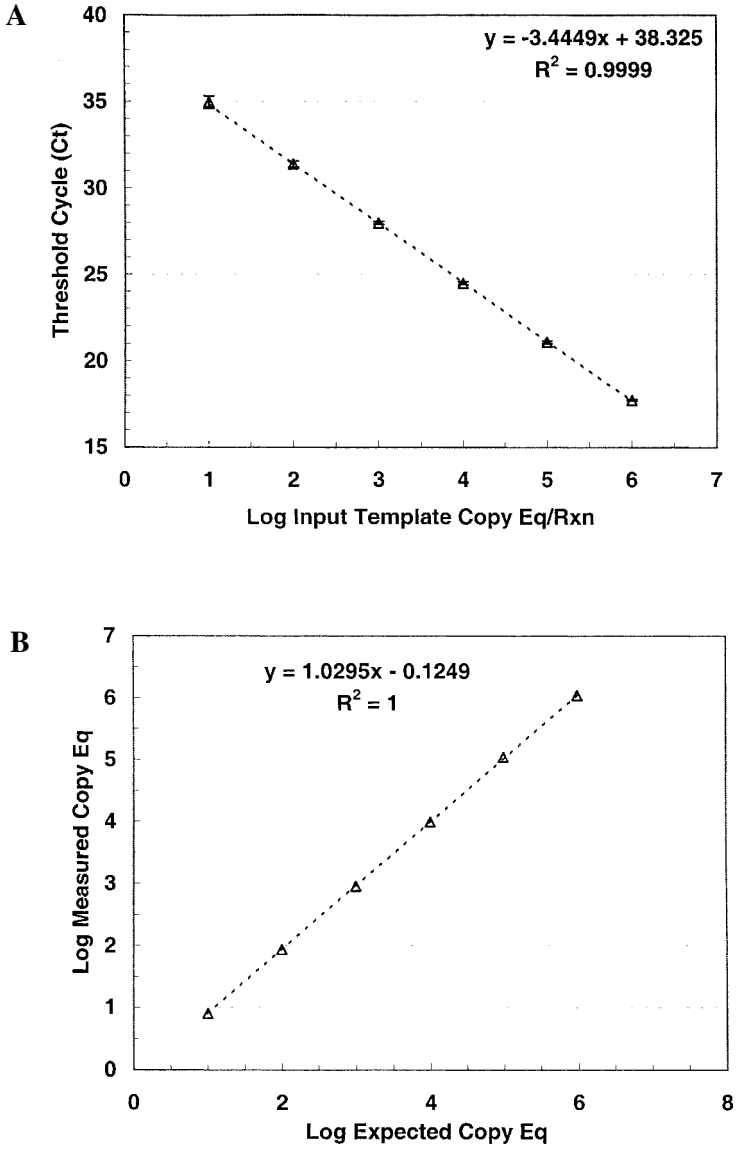

FIG. 2. (**A**) Plot of input control template (pMT2 plasmid) copy number versus C_t value demonstrates assay precision and broad dynamic range. Plotted values represent mean values ± 1 SD for triplicate determinations in six independent experiments. (**B**) Plot of log expected copy equivalents per reaction versus log measured copy equivalents per reaction demonstrates precision and accuracy of the assay, along with dynamic range. Plotted values represent mean values for averaged triplicate determinations in six independent experiments, performed and analyzed as described in Materials and Methods. Average interassay percent coefficient of variation (%CV) was 14% (range, 7–24%) over the input template range of 10^6 copy Eq/reaction to 10^1 copy Eq/reaction, with a trend for greater variation at lower template copy numbers.

mocytometer (viable and non-viable; average viability >80% by trypan blue exclusion), then pelleted for DNA extraction. For each sample, DNA was prepared from 1×10^3 to 1×10^6 cells using the PureGene kit, essentially according to manufacturer recommendations (Gentra Systems, Minneapolis, MN).

Specimens for analysis

To evaluate interassay precision, data were pooled from control template standard curves from six experiments, with each curve covering a nominal input template copy number range from 1.0×10^0 to 1.0×10^6 copy equivalents/reaction. For analysis of interassay precision and accuracy, six separate experiments were performed in which dilutions of the control target template used for generation of standard curves were prepared, then tested as unknowns in triplicate at each chosen input template copy number. These latter samples were tested in experiments in which copy numbers were experimentally determined on the basis of a standard curve constructed from an independent dilution of the control template. The overall mean average values and percent coefficient of variation (%CV) for interassay variation were then determined at each nominal copy number, on the basis of data obtained in the separate experiments.

To evaluate the impact of background DNA on the sensitivity and accuracy of quantitation, and to approximate the specimen background matrix for typical specimens, we compared standard curves generated on the basis of a dilution series of plasmid pMT2 in nuclease-free water, or the same dilution series of plasmid in a background of nuclease-free water containing 1×10^5 cell equivalents of total DNA from the HTLV-I-negative murine hybridoma cell line 4D1. In separate studies, we evaluated the impact of including DNA from 1×10^6 HTLV-I human PBMCs per reaction on the sensitivity and accuracy of quantitation of diluted plasmid standards.

To evaluate interassay precision in a format that also reflected variation attributable to the nucleic acid extraction step for cellular samples, we used the HTLV-I provirus-containing cell line MT-2.[49] Replicate aliquots were prepared of serial dilutions of MT-2 cells, made into increasing numbers of cells of the HTLV-negative murine hybridoma cell line 4D1,[50] such that the total cell number remained constant at 1×10^6 cells (MT-2 plus 4D1) per aliquot. Replicate aliquots of each dilution were extracted and the DNA tested in triplicate by real-time PCR, in 10 independent experiments, as described above.

To confirm the specificity of the assay, we tested PBMC samples from 14 untreated HTLV-I-seronegative Jamaican patients with hematolymphoid malignancies (ages 48–78 years, 10 males and 4 females, 7 with a primary diagnosis of B cell non-Hodgkin's lymphoma and 7 with chronic lymphocytic leukemia). In addition, we also tested six PBMC samples from HIV-1^+, HTLV-I,II-seronegative subjects and 12 HIV-1^+, HTLV-I-seropositive coinfected subjects (6 males and 6 females, mean age 44.6 ± 12.7 years [SD], range 27 to 70 years)

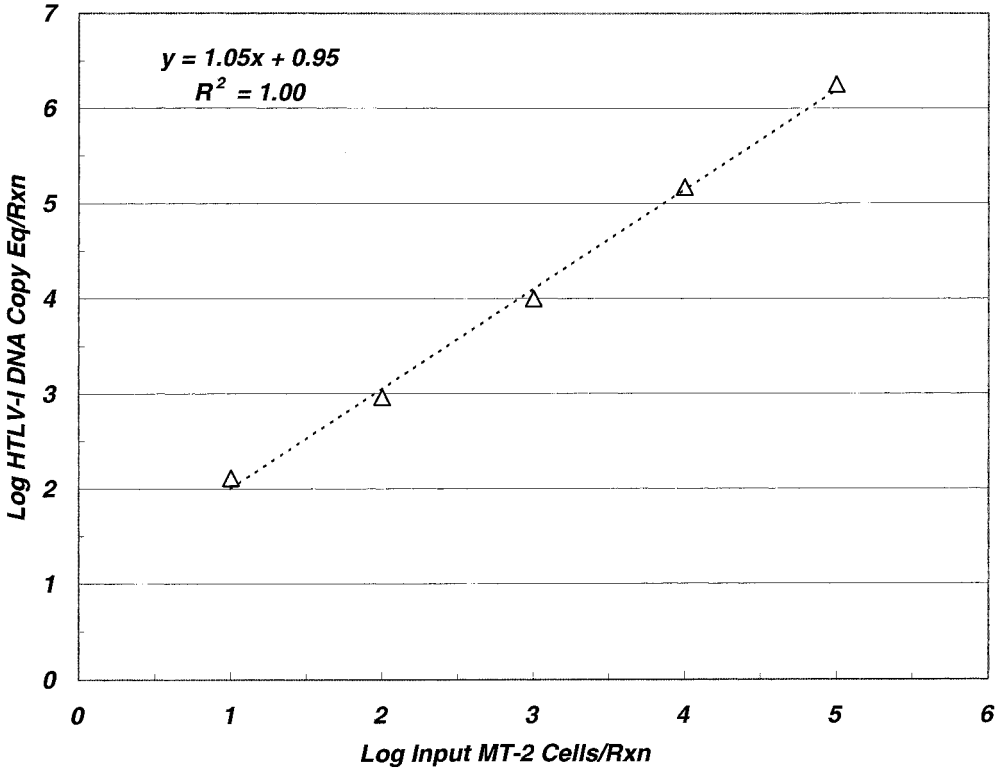

FIG. 3. Results for 10 independent extractions of replicate aliquots of the indicated number of MT-2 cells, mixed with sufficient HTLV-I-negative 4D1 cells to yield a constant background of 1×10^6 cells total DNA per sample. Each reaction used 10% of the total DNA, or 1×10^5 total cell equivalents. Values plotted are means of triplicate determinations in each of the 10 separate experiments (%CV values averaged 27% over the range from 1×10^5 MT-2 cells/reaction to 1×10^1 MT-2 cells/reaction).

attending a sexually transmitted disease clinic in Trinidad, not receiving antiretroviral treatment at the time the specimens were procured.[51]

To demonstrate the utility of the assay to quantify viral load in clinical specimens from patients with symptomatic HTLV-I-associated disease, we analyzed PBMC specimens from two patients with ATLL. For each sample, three replicate aliquots were separately extracted and analyzed with triplicate amplifications for each extracted sample in each of the four experiments. DNA from the patient derived cellular specimens was also analyzed to quantify copy number for the endogenous ERV-3 sequence, to allow normalization of results, on the basis of two copies of the ERV-3 sequence per diploid cell.[47]

We also compared results obtained by real-time PCR with values for replicate aliquots of a panel of specimens, determined previously by a quantitative competitive (QC)-PCR procedure, as described,[32] or seven HTLV-I-infected patients with HAM/TSP. Patients were from the Caribbean basin ($n = 2$), Papua New Guinea ($n = 1$), and the United States ($n = 4$), and on the basis of serological testing had been infected with HTLV-I for between at least 2 and 10 years at the time samples were obtained. Finally, we evaluated a series of longitudinal specimens in two patients with HAM/TSP to assess the composite variability attributable to both biological and analytical variation.

RESULTS

Analyses were performed to evaluate the dynamic range and precision of quantitation for the pMT2 control template. Figure 2A shows a plot of C_t values versus log nominal input template copy number, demonstrating the dynamic range and precision of the assay. Ten copy equivalents per reaction was readily detected; single-copy detection was achieved with a frequency of 29% (13 of 45 reactions, in 15 separate assays), consistent with statistical expectations. The linear dynamic range was at least 5 logs. Figure 2B demonstrates the accuracy and precision of the assay, showing good agreement between expected and determined values for independent serial dilutions of control templates, with an average interassay coefficient of variation of 14%. As expected, there was a trend for greater variability at lower copy numbers, presumably reflecting the effects of stochastic phenomena operative at low input template copy number. The presence or absence of background DNA equivalent to 1×10^5 cells per reaction did not affect the sensitivity or accuracy of quantitation of plasmid control templates, as results were comparable to those obtained in the absence of background DNA, within 13% (data not shown).

Results for testing of serial dilutions of the MT-2 cell line, in a constant background of total DNA from 1×10^6 cells per extracted sample, are shown in Fig. 3. The determined values show good reproducibility at each nominal MT-2 cell number, and excellent proportionality of measured results, consistent with the experimental dilution factors. The results provide a consistent nominal copy number of 13.0 ± 3.6 HTLV-I *tax* DNA copies per MT-2 cell (range, 9.2–18) across a 4-log range of input MT-2 cells. This approximates results reported previously using earlier methods.[52]

The specificity of detection of HTLV sequences by this assay system was demonstrated by testing of PBMC specimens from 14 HTLV-I-seronegative patients with hematolymphoid malignancies; none showed evidence of HTLV-I DNA sequences (assay threshold, 10 copy Eq/10^5 cell equivalents DNA) (Fig. 4). In testing of PBMC samples from 6 HIV-1^+, HTLV-I,II-seronegative patients, and 12 HIV-1^+, HTLV-I-

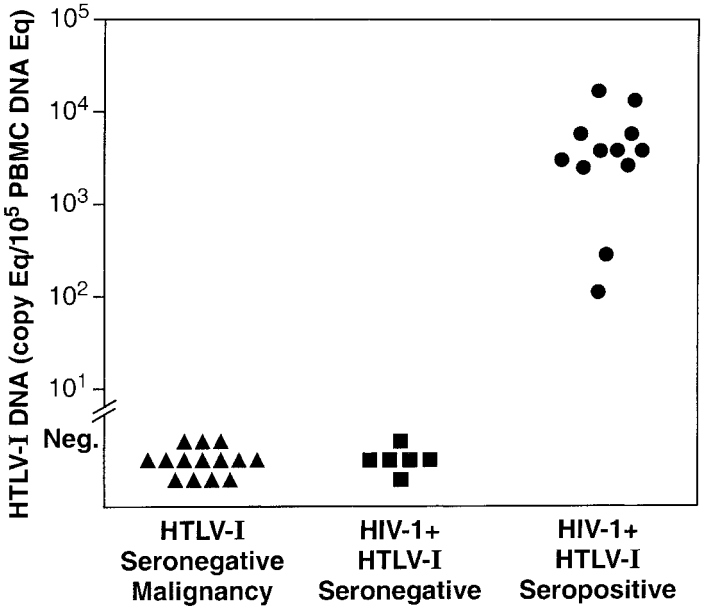

FIG. 4. Assay specificity. Results for HTLV-I,II-seronegative subjects with hematolymphoid malignancies; HIV-1-infected, HTLV-I,II-seronegative subjects; and HIV-1, HTLV-I-coinfected subjects are summarized. Values shown are normalized HTLV-I copy Eq/10^5 cell equivalents, based on ERV-3 measurements of the same samples. Threshold sensitivity for the assay is 10 HTLV-I copy Eq/10^5 PBMC DNA equivalents.

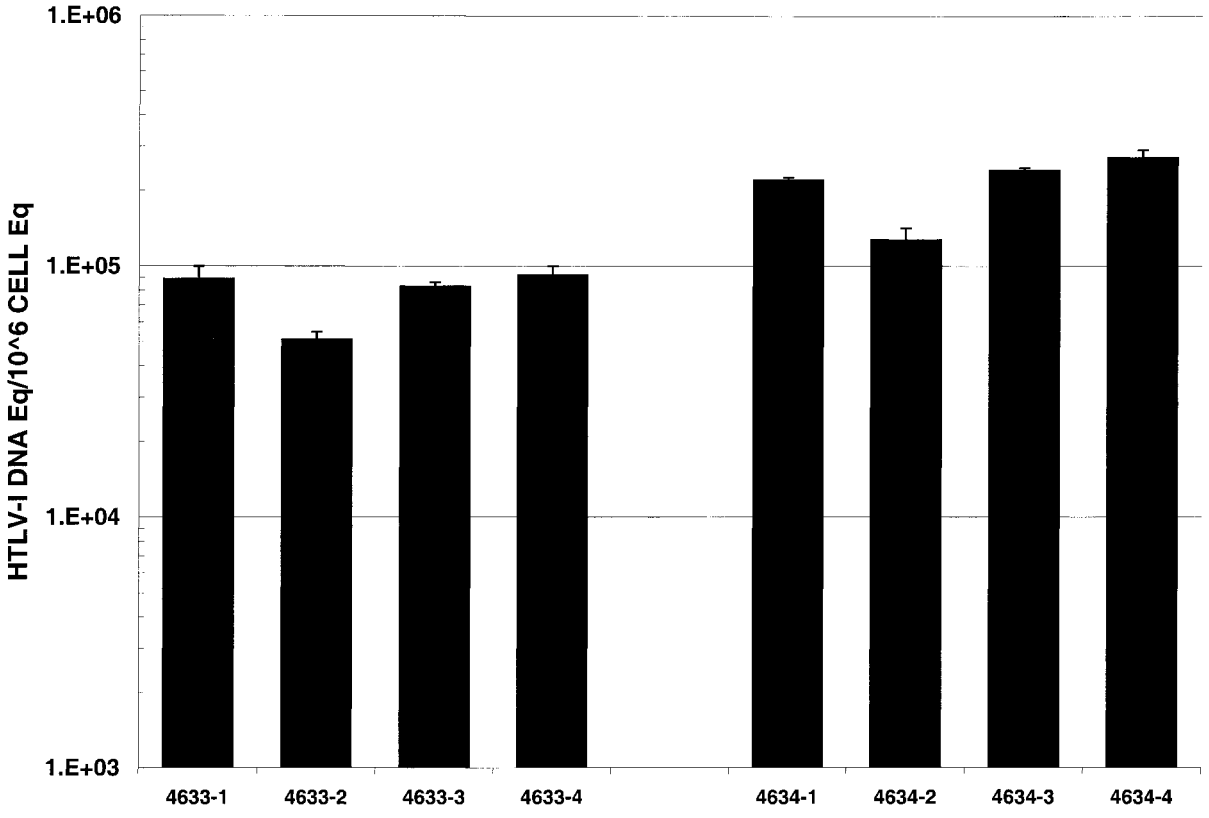

FIG. 5. Assay reproducibility for four independent extractions of replicate PBMC aliquots from two HTLV-I-infected patients, with quantitative analysis in triplicate (mean ± 1 SD) for each separate extraction. Results presented are normalized on the basis of parallel determination of ERV-3 copy numbers in the same extracted DNA samples. The average interassay %CV was 23% for patient A, and 27% for patient B.

seropositive coinfected patients attending a clinic for sexually transmitted diseases in Trinidad, quantifiable levels of HTLV-I DNA were measured in all 12 HTLV-I-seropositive patients and in none of the HIV-1^+, HTLV-I,II-seronegative patients (Fig. 4).

Figure 5 shows results obtained by using the assay, with normalization for ERV-3 to control for DNA recovery, to analyze four replicate aliquots of PBMCs from each of two HTLV-I-infected patients with ATLL. The results yield an average interassay coefficient of variation of 25%. Analysis of the within-assay variation for triplicate amplifications for each separate extracted DNA specimen yielded an average intraassay coefficient of variation of 7% (range, 1–14%), suggesting that the majority of overall interassay variation observed is due to sources of variation other than those inherent in the PCR amplifications themselves. Normalization based on parallel measurement of ERV-3 in the same samples reduced the average interassay variation for these samples from 31 to 25% (data not shown).

As shown in Fig. 6, in separate studies of 14 specimens from 7 HTLV-I-infected patients with HAM/TSP, the results obtained with the real-time PCR assay showed good overall agreement with results from a previous analysis of replicate aliquots of the same specimens, using a quantitative-competitive PCR (QC-PCR) method.[32] While there is not an exact one-to-one concordance between the two sets of values, this is not surprising since the measurements were each calibrated to independent reference preparations of control template. There was, however, an overall good correlation between the values determined by the two techniques (Spearman rank correlation coefficient for analysis of log transformed data, $R = 0.8132$, $p < 0.0007$).

Figure 7 demonstrates that on the basis of analysis of sequential specimens in two clinically stable patients, PBMC viral load levels were comparatively stable over approximately 4 years of follow-up.

DISCUSSION

We took advantage of recently available instrumentation to develop an assay for the sensitive and reliable quantification of HTLV-I DNA in PBMC samples, on the basis of real-time quantitative detection of PCR product generation. The assay described has good specificity, with no false-positive results detected in testing of 20 samples from HTLV-seronegative subjects, and no cross-reactivity with HIV-1, allowing its use in testing specimens from coinfected patients. The assay has excellent performance characteristics, providing outstanding sensitivity, an extremely broad dynamic range, and good precision,

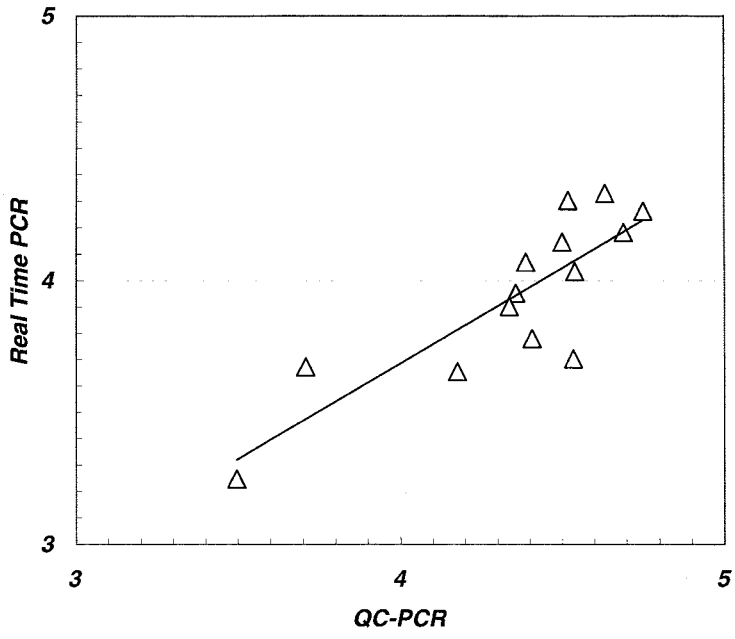

FIG. 6. Comparison between PBMC DNA copy number as determined by a QC-PCR assay[32] and DNA copy numbers determined by real-time PCR (Spearman rank correlation coefficient for analysis of log transformed data, $R = 0.8132$, $p < 0.0007$).

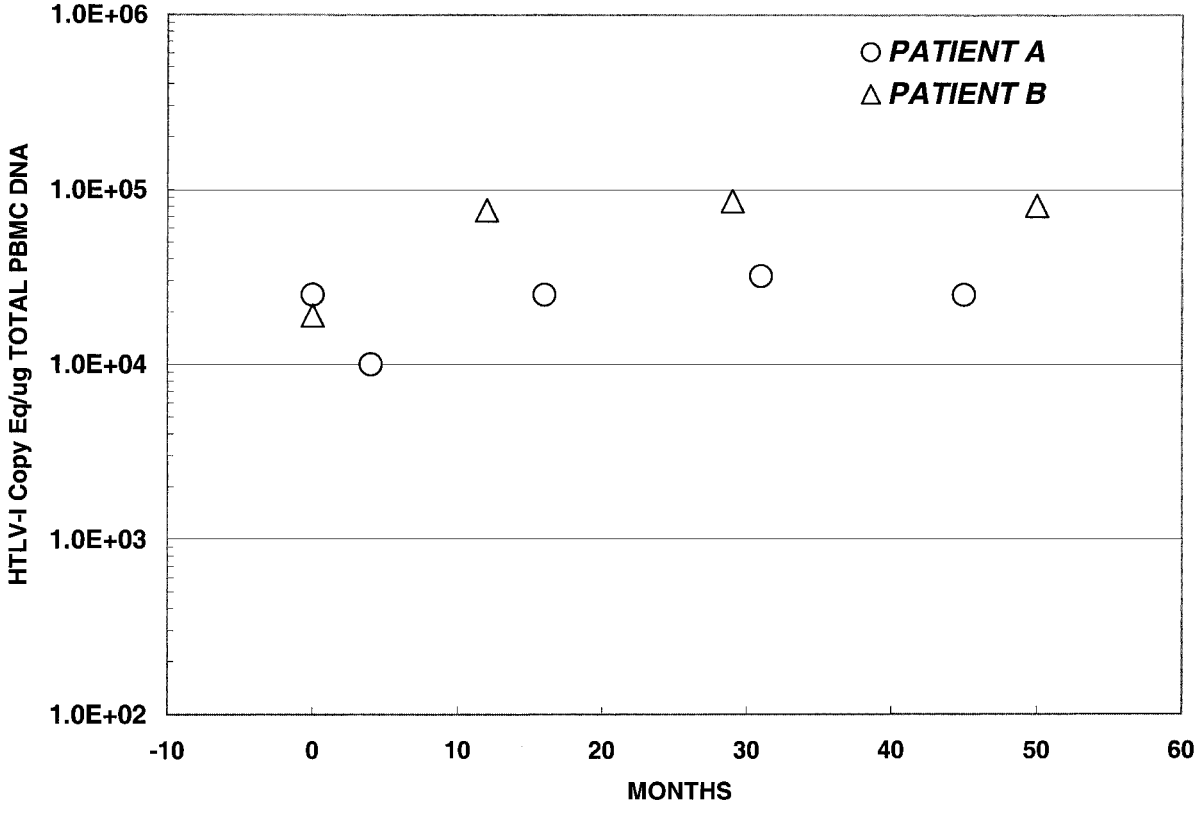

FIG. 7. Longitudinal stability of PBMC viral load in two HTLV-I-infected patients with HAM/TSP, with serial specimens covering approximately 4 years.

comparable to that of previously available benchmark methods such as QC-PCR approaches.[32] The assay format eliminates the need for manipulation of amplified products, a major source of potential contamination, and represents a significant improvement in both throughput and convenience over previous methods. The reagents used in the assay are designed to take advantage of sequences conserved across described isolates of HTLV-I and HTLV-II, and allow quantification of both HTLV-I and HTLV-II DNA. Indeed, in separate studies we demonstrated that the inosine substitutions incorporated into the primers used allowed accurate quantitation of both HTLV-I and HTLV-II plasmid control standards, while the use of exactly matched primers allowed accurate quantitation of the matched viral sequence (either HTLV-I or HTLV-II) but marked underquantitation of the mismatched sequence (W. Miley, unpublished data).

The parallel determination of copy number for ERV-3[47] allowed normalization of assay results to provide a control for any inaccuracies or inconsistencies in cell-counting procedures, differences between specimens in nucleic acid recovery, or potential differences in efficiency of different PCRs in the same assay run, as can be observed when substances inhibitory to the PCR copurify with the extracted DNA, providing an additional level of reliability to the measurements. In the present studies, use of ERV-3 normalization did reduce interassay variation in results obtained for testing replicate aliquots of the same specimens.

The present studies were intended simply to establish the performance characteristics of the assay, and to establish the feasibility of using the assay to measure viral load in typical clinical specimens, rather than to address clinical issues. However, both the present and future versions of this assay, and corresponding RT-PCR assays for quantification of cell-free and cell-associated HTLV-I and HTLV-II RNA, should be useful in further clarifying the relationship between viral load and viral replication and the pathogenesis of HTLV-I- and HTLV-II-associated diseases. Indeed, a study using a similar real-time PCR format assay for HTLV-I DNA suggested that high proviral load in PBMCs was associated with a higher incidence of HAM/TSP among HTLV-I-infected subjects.[53] Such assays should also be extremely useful in the rapid evaluation of antiviral and other approaches to the treatment of HTLV-I- and HTLV-II-associated diseases, as they are developed.

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